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SO JOURNAL OF ANIMAL SCIENCE, (1993 Mar) 71 (3) 687-93.

L4 ANSWER 49 OF 66 MEDLINE DUPLICATE 17
AU Kolb A F; Albang R; Brem G; Erfle V; Gunzburg W H; Salmons B
TI Characterization of a protein that binds a negative regulatory element in the mammary-specific whey acidic protein promoter.
SO BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (1995 Dec 26) 217 (3) 1045-52.

L4 ANSWER 50 OF 66 MEDLINE DUPLICATE 18
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TI The effect of various introns and transcription terminators on the efficiency of expression vectors in various cultured cell lines and in the mammary gland of transgenic mice.
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The effect of various introns and transcription terminators on the efficiency of expression vectors in various cultured cell lines and in the mammary gland of transgenic mice

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Abstract

Various combinations of promoters, introns and transcription terminators were used to drive the expression of bovine growth hormone (bGH) cDNA in different cell types. In constructs containing the human cytomegalovirus (hCMV) promoter and the SV40 late genes terminator, the intron from SV40 late genes (VP1) was much more efficient than the intron from the early genes (t). The synthetic intron SIS generated by the association of an adenovirus splice donor and an immunoglobulin G splice acceptor showed the highest activity. The respective potency of these introns was similar in several mammalian (CHO, HC11 and COS) and fish (TO2 and EPC) cells. The rabbit whey acidic protein (WAP) gene promoter was highly efficient to drive the expression of bGH gene in the HC11 mammary cell lines. In contrast, the bGH cDNA under the control of the same promoter was much less efficiently expressed when the SV40 VP1 intron and transcription terminator were used. The rabbit WAP gene and the human GH gene terminators did not or only moderately enhanced the expression of the construct WAP bGH cDNA. Introduction of a promoter sequence from the mouse mammary tumor virus (MMTV) LTR in the VP1 intron increased very significantly the expression of the WAP bGH cDNA. Although several of these vectors showed high potency when expressed stably in HC11 cells, all of them were only moderately efficient in transgenic mice. These data indicate that the VP1 and the SIS introns may be used to express foreign cDNAs with good efficiency in different cell types. The addition of an enhancer within an intron may still reinforce its efficiency. However,

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transfection experiments, even when stable expression is carried out, are poorly predictive of the potential efficiency of a vector in transgenic animals.

Keywords: Intron; Terminator; Transcription; Cell; Transgenic

1. Introduction

The efficiency of transgenes is in most cases largely unpredictable. Native genes containing all or part of their introns and their terminators are generally much better expressed than the corresponding cDNAs. A systematic study carried out several years ago by Brinster et al. (1988) and Palmiter et al. (1991) indicated that the presence of introns is greatly beneficial for all transgenes but not for all gene constructs expressed in cultured cells. In these studies, several promoters introns and transcription terminators were used. Although some of the combinations were highly efficient to express foreign cDNAs in transgenic mice, no general rules to generate potent transgenes have been defined. In the present work, the efficiency of several combinations of introns and transcription terminators have been compared using in all cases bGH cDNA as the reporter gene in different cell types. This study was undertaken to tentatively define potent vectors for transgenics. Such vectors are definitely needed in different cases, when a high level expression of antisense RNA or the production of large amount of rare recombinant protein in the milk of transgenic animals are required (Houdebine, 1994). For this purpose, transient transfection assays were carried out in different cell types using the human cytomegalovirus promoter to evaluate the efficiency of various introns. To tentatively evaluate the intrinsic efficiency of introns, not only a mammary cell line but several mammalian, bird and fish cells were used. In addition, the effect of transcription terminators was measured in vectors containing the rabbit WAP gene promoter in the HC11 mouse mammary cell lines. In a previous work (Thépot et al., 1995) it was observed that the native bovine GH gene was highly expressed in milk of transgenic mice when driven by the rabbit WAP gene promoter. In the present study three vectors containing the same promoter but the bGH cDNA instead of the whole gene and which showed high potency in HC11 cells were tested in transgenic mice.

2. Materials and methods

2.1. Gene constructs

The human cytomegalovirus early gene promoter was introduced in the *Pvu*II site of the pSVL vector (Pharmacia; Hernandez-Betancourt et al., 1993) which contains the SV40 late genes promoter, intron (VP1) and transcription terminator. The bGH cDNA was always introduced in the *Sma*I site of the polylinker located after the VP1 intron. The fragment *Eco*RI-*Sal*I of this plasmid was excised and cloned in the polylinker of pPoly III p1.

The *Kpn*I-*Xba*I fragment of the pPoly III p1 was removed and replaced by the VP1 intron (Hernandez-Betancourt et al., 1993), t intron or by the SIS intron from pML SIS CAT (Choi et al., 1991). Alternatively, the t intron was added after the bGH cDNA in the *Sac*I site.

The rabbit WAP gene promoter 6.3 kb (Devinoy et al., 1991) cloned in pPoly III plasmid was associated with the pSVL fragment containing the SV40 late genes intron and terminator and the bCH cDNA. The *Bam*H1-*Sal*I fragment from pSVL containing the SV40 late genes terminator was excised and several other terminators were introduced. These terminators were from the rabbit WAP gene (a 190 bp and a 4800 bp fragment) and from the human GH gene (a 800 bp fragment in the 3′ region containing the last exon and the terminator.

The MMTV LTR fragment between positions −201 and −49 was obtained by PCR (Buetti, 1994). It was introduced in the *Eco*RV site of the VP1 intron.

All these constructs are described in Fig. 1.

The drawings are not on scale and do not respect the size of the various DNA fragments.

2.2. Cell culture and transfection

The mammalian cell lines used were CHO, COS 7, HCII, the fish cell lines TO2 and EPC were those used in our previous work (Hernandez-Betancourt et al., 1993).

The various cell types were transfected using the conventional calcium phosphate precipitation or lipofection using Lipofectin (BRL).

For transient expression in 60-mm diameter dishes, 3 to 5 μg of each plasmid were used with calcium phosphate and 3 μg with Lipofectin. Two days later, medium was collected and kept frozen until assay.

For the generation of stable HC11 clones in

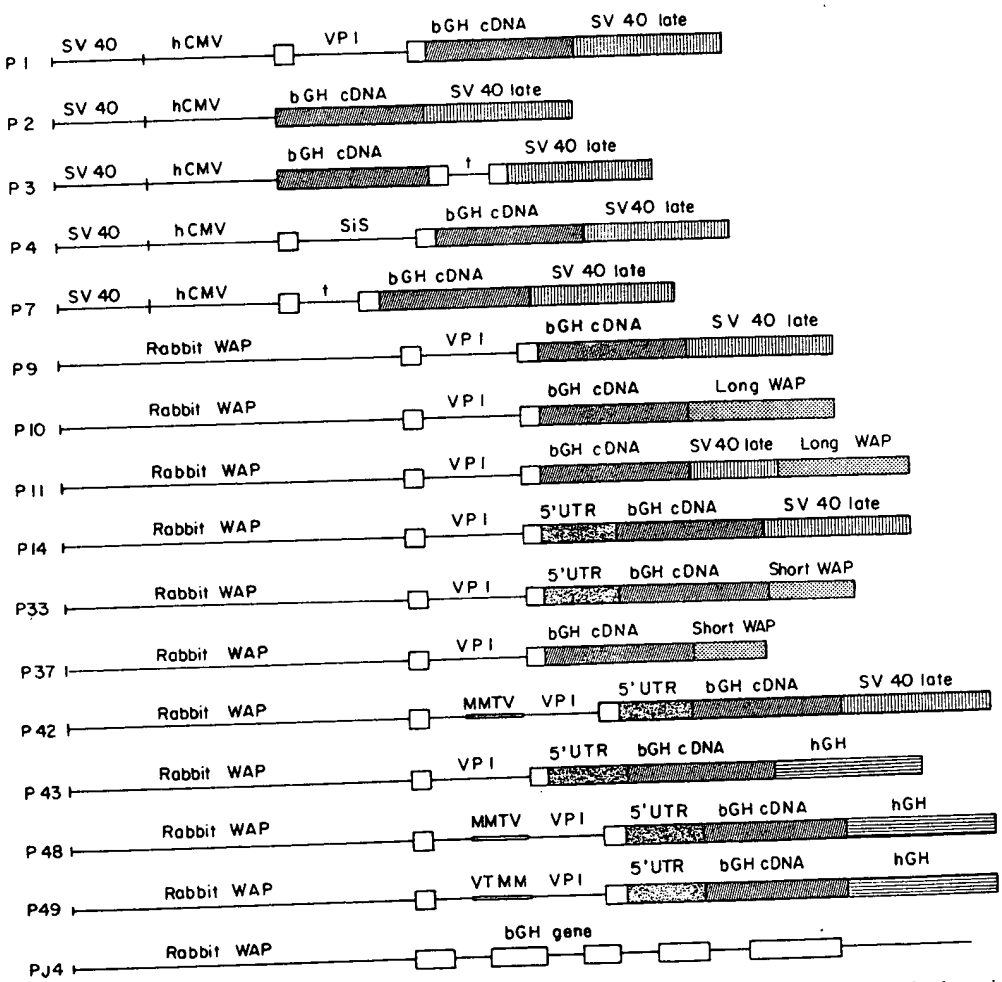

Fig. 1. Schematic representation of the various gene constructs. SV40 is the SV40 late genes enhancer present in the original pSVL vector (Pharmacia). hCMV is the promoter and enhancer from the human cytomegalovirus early genes. VP1 intron is the SV40 late genes intron, present in the original pSVL vector. bGH cDNA is the intronless coding sequence of bGH gene. SV40 late is the SV40 late genes terminator, present in the original pSVL vector. Short and long WAP are the rabbit WAP gene terminator (190 bp and 4800 bp, respectively). hGH is the 800 pb fragment in 3' of the last exon containing all the human GH terminator. t intron is the SV40 early genes intron. SIS intron is the hybrid intron containing an adenovirus splice donor and an immunoglobulin G splice acceptor. bGH gene is the genomic fragment containing the whole functional gene. 5' UTR is an untranslated region which will be described elsewhere. MMTV is the LTR fragment from the mouse mammary tumor virus (-201 to -49).

100-mm diameter dishes, 8 μg of the various plasmids and 2 μg of the pRSV neo were used with Lipofectin. Clones were selected using G-418 (200 $\mu g\ ml^{-1}$). The mixed clones were amplified and cultured in a medium containing EGF (10 ng ml^{-1}) until confluency. They were then cultured for 4 more days under the same conditions until hyperconfluency was obtained (Ball et al., 1988). A new medium containing no EGF, but supplemented with insulin (5 $\mu g\ ml^{-1}$), dexamethasone (10^{-6} M) and ovine prolactin (NIH PS13) (5 $\mu g\ ml^{-1}$), was added. After 2 d, the media were collected and frozen until use. HC11 cells are highly dependent on their degree of hyperconfluency to be sensitive to the lactogenic hormones. Experiments carried out repeatedly indicated that the same level of stimulation could not be obtained in all cultures for the endogenous β-casein gene. This explains why the same gene constructs appear to have a quite different efficiency in the various experiments reported in the present work. A comparison of the gene constructs efficiency was therefore possible only within a given culture using different plasmids transfected simultaneously.

2.3. Generation of trangenic mice

The inserts containing no more plasmid sequences were injected into mouse pronuclei ($\cong$ 500 copies). The transgenic animals were identified using a rapid PCR method (Attal et al., 1995). Milk was collected after injection of oxytocin (Devinoy et al., 1994).

2.4. Measurements of bGH

The bGH concentrations in the culture medium and milk from transgenic mice were determined by radioimmunoassay using anti bGH antibody from NIH. Controls were the medium of non transfected cells. The low level of bGH in the control corresponded to the endogenous hormone present in the calf serum added to the culture medium.

2.5. Measurement of bGH mRNA concentration

Total RNA was extracted from frozen cells using the guanidinum isothiocyanate-phenol-chloroform method. RNA was separated on agarose gel, transferred to nylon membrane and hybridized with ^{32}P-labelled bGH cDNA (10^6 cpm ml^{-1}, 10^8 cpm μg^{-1} DNA). All these methods were used in our previous work (Bearzotti et al., 1992; Hernandez-Betancourt et al., 1993).

3. Results

3.1. The effect of introns

In a previous work, it was observed that, in CHO cells, the reporter chloramphenicol acetyl

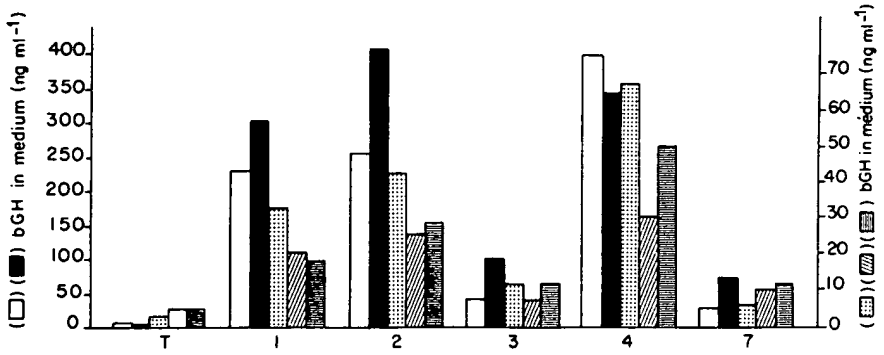

Fig. 2. Efficiency of various introns in different cell types. The different gene contructs are described in Fig. 1. The numbers correspond to these of each gene construct. T is the control medium of non-transfected cells. Results are the means of duplicates from two independent dishes. CHO (open bars); COS (solid bars); HC11 (dotted bars); TO2 (stripped bars); EPC (horizontal stripped bars).

transferase (CAT) gene was better expressed in the pSVL vector containing the hCMV promoter than in the conventional pCMV CAT containing the SV40 early genes intron and terminator (Hernandez-Betancourt et al., 1993). The vector was also efficient in the quail QT6 cell line, but not in TO2, CHSE and EPC fish cells. Deletion of the VP1 intron did not modify greatly the efficiency of the vector in CHO and QT6 cells. On the contrary, the vector without the intron was quite capable of expressing the CAT gene suggesting that the VP1 intron was not correctly spliced in fish cells, as opposed to the SV40 early genes intron.

In the present work, the same vector containing the hCMV promoter, the VP1 intron and terminator (p1; Fig. 1) was quite efficient in three mammalian cell line (CHO, HC11 and COS), in QT6 (not shown) and in TO2 and EPC (Fig. 2). Deletion of the VP1 intron enhanced somewhat the efficiency of the vector (Fig. 2). This result shows with no ambiguity that the VP1 intron is quite well processed when associated with bGH cDNA. Data not shown here indicated to us that this was also the case with the tilapia GH cDNA. The same was obviously not true with the reporter CAT gene (Hernandez-Betancourt et al., 1993).

The SV40 late gene region codes for several proteins VP1, VP2, VP3, resulting from alternative splicing. The generation of functional mRNA coding for VP2 and VP3 is therefore possible in the constructs containing the VP1 intron. These events are most likely of minor importance. Indeed, only one mRNA is observed with these constructs, suggesting that the splicing generating VP2 and VP3 is poorly efficient and negligible.

The SV40 early genes intron is very often added to expression vectors. It is then generally associated with the SV40 early genes terminator. This intron alone was added instead of the VP1 intron before and after the bGH cDNA to tenta-

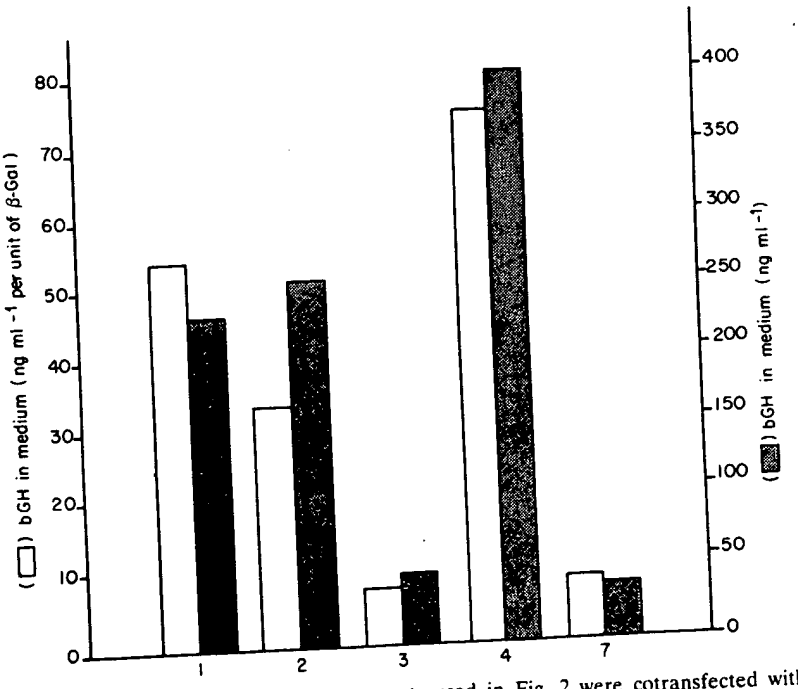

Fig. 3. Evaluation of transfection efficiency. The gene constructs used in Fig. 2 were cotransfected with the pCH 110 vector expressing the β-galactosidase gene. Results which are means of duplicates from two independent dishes are expressed in bGH concentration referred to β-galactosidase activity in the extracts from the same cells. ng ml^{-1} medium (shaded bars); ng per unit β-galactosidase (open bars).

tively evaluate its potency. This intron was in both cases much less efficient than the VP1 intron.

Several years ago, it was reported that the SIS intron generated by the association of an adenovirus and an immunoglobulin gene introns was highly efficient when added to vectors (Kaufman and Sharp, 1982; Choi et al., 1991). This intron was indeed much more potent than the SV40 early genes intron and slightly more potent than the VP1 intron (Fig. 2). This was particularly striking in the fish cells.

Interestingly, the hierarchy of the intron potency was essentially the same in the five tested cell lines.

The transfection efficiency was evaluated in one experiment reported in Fig. 3. The various plasmids were co-transfected with the pCH 110 plasmid which express the β-galactosidase gene under the SV40 early genes promoter. Results of Fig. 3 confirmed that the SIS intron was the strongest and the SV40 early genes intron the weakest intron.

3.2. The effect of terminators

The data reported above indicated that the SV40 late genes intron and terminator are of good efficiency, at least in cultured cells, for the expression of bGH cDNA. The same intron and terminator was therefore tested in the mammary HC11 cells using the rabbit WAP gene promoter. This promoter proved to be highly efficient to drive the expression of hGH (Devinoy et al., 1994), human α1-antitrypsin (Bischoff et al., 1992) and bGH genes (Thépot et al., 1995).

A comparison of the efficiency of the vectors containing the WAP gene promoter with either the whole bGH gene (pJ4) or the bGH cDNA with the VP1 intron combination revealed that the bGH gene was much more efficient than the cDNA in HC11 cells (Fig. 4).

The natural gene contains several introns and its own terminator. In order to evaluate the role of these elements, several terminators were compared to the SV40 late genes terminator. The SV40 late genes terminator region is known to

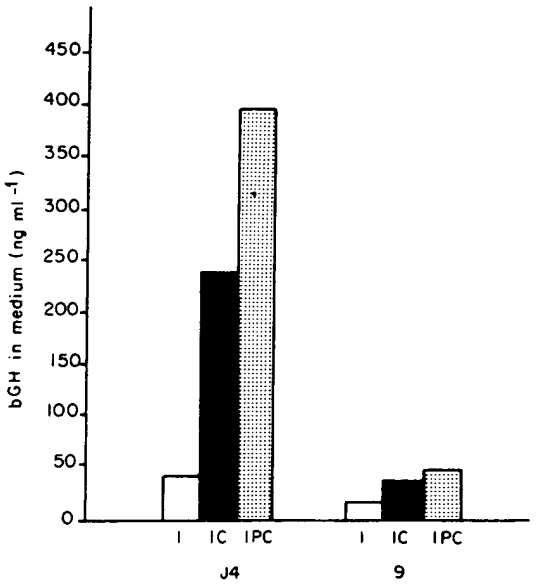

Fig. 4. Comparative efficiency of bGH gene and cDNA expression in HC11 cells under the control of the rabbit WAP gene promoter. The gene constructs used are described in Fig. 1. I, C and P designate insulin, dexamethasone and prolactin, respectively. bGH concentration (ng ml^{-1}) was measured in the culture medium of mixed stable clones after 2 d of hormonal induction. Results are the means of duplicates from two independent dishes issued each from two independent transfections.

contain a polyadenylation site and a well indentified termination site (Lutz and Alwine, 1994). The same is not true for most of the regions used as transcription terminators and in which only a polyadenylation site has been identified. The long rabbit WAP gene terminator alone or associated with the SV40 late genes terminator did not improve the expression of bGH cDNA in HC11 cells (Fig. 5).

In a recent work, it was shown that a short terminator from rat WAP gene was much more efficient than the long one to express the WAP gene in transgenic mice (Dale et al., 1992). The short rabbit WAP gene terminator was only slightly more efficient that the long one in HC11 cells (Fig. 5).

The hGH terminator (construct p43) was slightly more efficient than the other terminators (not shown).

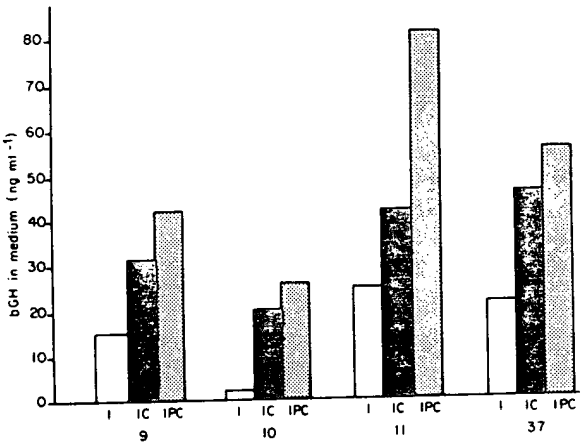

Fig. 5. Comparative efficiency of SV40 late genes, and rabbit WAP gene terminators. The induction of gene expression in HC11 cells was carried out as described in legend of Fig. 4. I, C and P designate insulin, dexamethasone and prolactin, respectively. The numbers correspond to the gene constructs described in Fig. 1. bGH concentration (ng ml^{-1}) in the media.

These data suggest that the terminator is not a limiting factor in the p9 vector.

3.3. Effect of the addition of an enhancer into intron

The first intron of several mammalian GH genes contains a functional glucocorticoid responsive element (GRE; Slater et al., 1985). In order to tentatively mimic the situation of the natural GH gene, a DNA fragment containing GRE was introduced in the VP1 intron (construct p42). The fragment used was part of the MMTV-LTR. This fragment contained four GRE, one NF1 and one Oct I binding sites.

The addition of this MMTV fragment within the VP1 intron enhanced very significantly the expression of the bGH cDNA as judged by mRNA concentration (Fig. 6). The content of bGH in the culture medium of HC11 cells was also 3-times higher when the MMTV fragment was incorporated into the VP1 intron (Fig. 7). The MMTV fragment was totally inefficient when it was introduced in opposite direction (constructs p48 and p49; not shown).

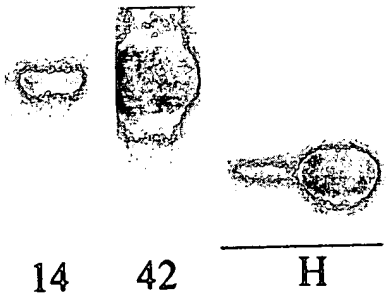

Fig. 6. Northern blot analysis of bGH mRNA. Total RNA was extracted from HC11 stable clones after an hormonal induction with insulin + dexamethasone and prolactin. The amount of total cellular RNA per lane was 10 μg. 14 and 42 correspond to the gene constructs described in Fig. 1: insulin + dexamethasone + prolactin. Total RNA from bovine hypophysis (H) was used as control (0.2 μg and 1 μg). The hybridized material was of higher size in the case of the vectors, due to the presence of extra sequences around the bGH cDNA.

3.4. The expression of vectors in transgenic mice

The vector containing the whole bGH gene (pJ4) proved to be highly expressed both in vivo

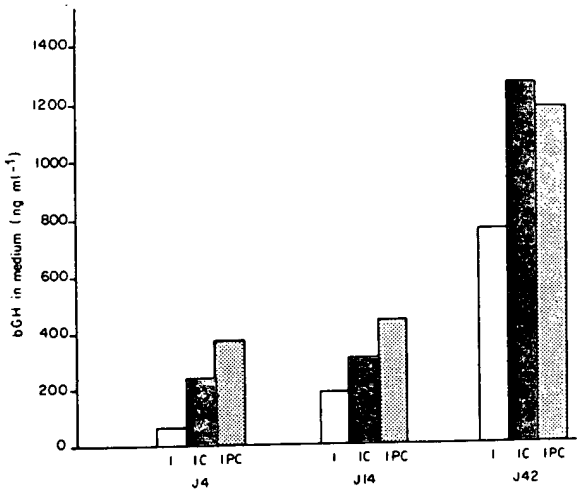

Fig. 7. The effect of an enhancer introduced into the VP1 intron. The −209 −49 MMTV fragment was introduced in the *Eco*RV site of the VP1 intron (Fig. 1). The comparative efficiency of the constructs containing or not the MMTV DNA was tested an HC11 cells. Conditions of culture are those described in legends of Fig. 4. I: insulin; C: dexamethasone; P: prolactin. The numbers correspond to the gene constructs described in Fig. 1. bGH concentration (ng ml^{-1}) in the media.

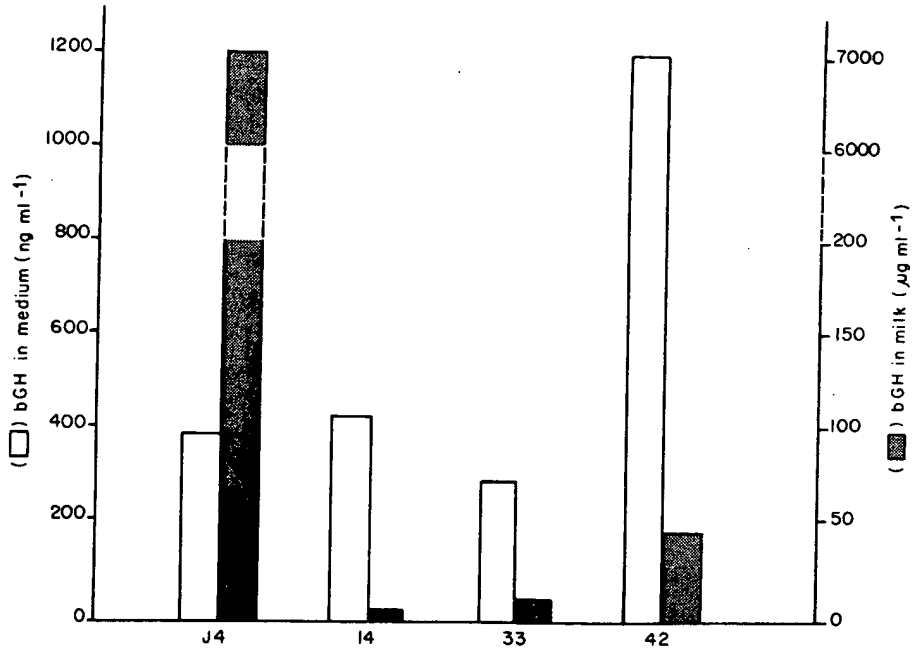

Fig. 8. Comparative efficiency of gene construct efficiency in HC11 cells and in transgenic mice. Results are the means of several independent lines of transgenic mice (these animals will be described with more details elsewhere). The numbers correspond to the gene constructs described in Fig. 1. bGH concentration in the media (ng ml^{-1}); in the milk ($\mu g\ ml^{-1}$).

(Thépot et al., 1995) as transgenes and in HC11 cells (Fig. 4). The p42 vector showing the highest efficiency in HC11 cells was tested in transgenic mice. The pJ4 led to a mean expression of more than 6 mg ml^{-1} milk in several transgenic mouse lines (Thépot et al., 1995). The p42 was much less efficient. The amount of the hormone found in milk of transgenic ranged from 1.5 to 280 $\mu g\ ml^{-1}$ only (Fig. 8). Thus, although p42 was significantly more efficient in HC11 cells than pJ4, it remained much weaker in vivo.

4. Conclusion

The present study revealed that the VP1 intron is almost as efficient as the SIS intron in various cell types. Unexpectedly, this intron was perfectly active in fish cells, a fact which was not observed with the reporter CAT gene (Hernandez-Betancourt et al., 1993). The reason of this discrepancy is unknown. An interference specific of the CAT gene with the mechanism of intron processing probably occurs in fish cells but not in mammalian and bird cells.

It is interesting to note that the relative efficiency of the different tested introns was the same in all the cell types used. Each intron thus seems to have its intrinsic capacity to be processed by the splicing machinery. Such a difference of intron efficiency was observed in the bovine GH gene (J.J. Kopchick, personal communication). In the GH gene, the last intron is also not quantitatively processed giving rise to the generation of a GH molecule having an additional aminoacid sequence (Hampson et al., 1989).

The SV40 early genes intron is known to be processed with a low efficiency due to its too small length (Fu et al., 1988). The VP1 intron thus seems a better candidate to be used for the expression of foreign cDNA in trangenic animals.

In the cultured cells, the presence of the VP1

and SIS intron modified only moderately the expression of the bGH cDNA. This fact confirms that introns are not strictly required in vitro for gene expression.

Although efficient, the SV40 late genes intron and terminator (Carswell and Alwine, 1989) were unable to allow a high expression level of bGH cDNA under the rabbit WAP gene promoter in HC11 cells. The use of other terminators indicated with no ambiguity that this element was not a limiting factor, at least in vitro.

Interestingly, the addition of GRE in the VP1 intron markedly stimulated the expression of the bGH cDNA in HC11 cells. This addition mimicked to some extent the natural bGH gene which contains a functional GRE in its first intron. WAP gene expression is highly dependent on glucocorticoids (Devinoy et al., 1991). It is not known whether the amplificatory action the MMTV GRE results from a specific interaction with the WAP promoter or whether it would stimulate the expression of a construct having a promoter not responsive to hormones. In addition, the presence of NF1 and Oct1 in the MMTV promoter fragment used may also favour transcription (Buetti, 1994).

The vector p42 which expressed bGH in HC11 cells with a much higher efficiency than pJ4 which contains the whole bGH gene was far from being as potent in transgenic mice.

Unknown potent sequences favouring transgene expression are therefore present in the bovine GH gene but absent in the p42 vector. This data brings additional support to the idea that DNA sequences with specific and unknown mechanism are required to maintain a transgene in an active configuration. These sequences which remain to be found are obviously not required in cultured cells, even when the foreign genes are integrated. This confirms that the use of cultured cells is of low predictive value to define the potential efficiency of a gene construct for transgenic animals.

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Abstract

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